

In the Description

Please replace paragraph [0003] with the following re-written paragraph.

--The production of proteins such as insulin, GM-CSF (sargramostin; sold as Leukine®), and hirudin (lepirudin; sold as Refludan®) is an example of the successful development of genetic engineering processes which are based on the synthesis of the particular protein or precursors thereof in yeast. Generally, yeasts can directly synthesize hirudins with good yields, which are on the gram scale, when using *Hansenula polymorpha* (Weydemann et al., *Appl. Microbiol Biotechnol.* 44:377-385, 1995) or *Pichia pastoris* (Rosenfeld et al., *Protein Expr. Purif.* 4, 476-82, 1996).--

Please replace paragraph [0049] with the following re-written paragraph.

--The examples below describe the invention in more detail, without being restrictive.

EXAMPLE 1

Construction of an Expression Cassette Encoding a Fusion Protein Made of Leu-Hirudin (lepirudin; sold as Refludan®)-Arg-Mini-Proinsulin--

Please replace paragraph [0050] with the following re-written paragraph.

-- Starting materials were the plasmids pK152 (PCT/EP00/08537, which is incorporated by reference herein in its entirety), pSW3 (EP-A 0 347 781, which is incorporated by reference herein in its entirety) and the recombinant yeast plasmid

derivative coding for bovine interleukin 2, which is $\text{p}\alpha\text{ADH2}$ plus the cDNA for IL2 (Price et al., Gene 55, 1987, which is incorporated by reference herein in its entirety). The yeast plasmid was distinguished by carrying the α factor leader sequence under the control of the yeast ADH2 promoter. This sequence was followed by the bovine interleukin 2 cDNA sequence which was connected via a KpnI restriction enzyme recognition site and which contained, an NcoI restriction enzyme recognition site in the untranslated 3' end which was unique in the vector. Thus, the cDNA sequence was readily removable from the plasmid via KpnI/NcoI cleavage. Since good expression yields were reported, it was assumed that the remaining 3' interleukin 2 sequence (as a terminator sequence) had a stabilizing effect on the mRNA and thus need not be replaced by a yeast specific terminator sequence. Plasmid pK152 carried the DNA sequence coding for Leu-hirudin (lepirudin; sold as Refludan®) and plasmid pSW3 carried the DNA sequence for mini-proinsulin. The gene sequence encoding hirudin-Lys Arg-mini-proinsulin was first prepared by means of PCR technology. For this purpose, 4 primers were prepared with the aid of the Expedite™ DNA synthesis system:--

Please replace paragraph [0060] with the following rewritten paragraph.

-- This was then followed by 25 cycles of 30" at 95°C, 30" at 55°C and 30" at 72°C. The last cycle was followed by an incubation at 72°C for 3 minutes, and the reaction was subsequently stopped by cooling to 4°C. Since the primers hir_insrevkr and hir_insfkr were 100% complementary, the DNA products of the two products overlap according to said sequence so that in a third reaction under the same conditions as described above, using 5% of the PCR-fragments generated in the first two reactions as templates and the primers hirf1 and insnco1rev, a DNA fragment was formed, which encoded hirudin and mini-proinsulin separated by Arg. The PCR

fragment was digested according to the manufacturer's protocol by the enzymes KpnI and NcoI and then, in a T4 ligase reaction, inserted into the $\text{p}\alpha\text{ADH2}$ vector opened by KpnI/NcoI. In the same manner, except as noted below, as Example 7 of EP-A 0 347 781, which is incorporated by reference herein in its entirety, competent *E. coli* MM294 cells were then transformed with the ligation mixture. Plasmid DNA was then isolated from two clones for characterization by means of DNA sequence analysis. After confirmation of the inserted DNA sequence, DNA of a plasmid preparation was used to transform cells of baker's yeast strain Y79, according to said Example. However, when using the $\text{p}\alpha\text{ADH2}$ vector, introduction of the vector was followed by selecting for complementation of the *trp1-1* mutation on yeast minimal medium agar plates, which contained no tryptophan, in contrast to said Example. For another control, plasmid DNA was reisolated from yeast transformants and analyzed by means of restriction analysis by standard techniques. The expression vector constructed was denoted pADH2Hir_Ins . Expression was carried out according to Example 4 of the present document. The fusion protein was found in the supernatant.

EXAMPLE 2

Construction of an Expression Cassette Encoding a Fusion Protein Made of Leu-Hirudin (lepirudin; sold as Refludan®)-Gly Asn Ser Ala Arg-Mini-Proinsulin--

Please replace paragraph [0075] with the following re-written paragraph.

--The hirudin concentration was determined according to the method of Grießbach et al. (Thrombosis Research 37, pp. 347-350, 1985, which is incorporated by reference herein in its entirety). For this purpose, a lepirudin Refludan® standard was included in the measurements in order to establish a calibration curve from which

the yield in mg/l could be determined directly. The biological activity, as measured in accordance with the method of Grießbach et al., was also a direct measure for correct folding of the proinsulin component of the fusion protein. Alternatively, although not performed in this Example, it is possible to use a proteolytic *Staphylococcus aureus* digestion and subsequent analysis in an RP-HPLC system to determine the correct S--S bridge formation.

EXAMPLE 6

Purification of the Fusion Protein--